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THE IDENTIFICATION OF THE RIBOSOMAL RNA CISTRON BY SEQUENCE COMPLEMENTARITY, II. SATURATION OF AND COMPETITIVE INTERACTION AT THE RNA CISTRON*

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The presence of a sequence in DNA complementary to homologous ribosomal RNA is indicated by experiments described in a preceding paper.[1] The data showed that a hybrid complex resistant to RNAase is formed when mixtures of ribosomal RNA and denatured DNA from the same cell are heated and then subjected to a slow cool.[2] When DNA from a heterologous source is substituted, no such complex is observed. The specific pairing of ribosomal RNA with a complementary sequence in DNA leads to the following two predictions: (1) The ratio of RNA to DNA in the specific complex should approach a maximum value at levels indicating the involvement of a minor fraction of the DNA; (2) Nonribosomal RNA should not compete for the same site.

Experiments relevant to these two issues have been performed. It is the intent of the present paper to describe the results obtained and discuss their implications. The data are consistent with the existence of a specific sequence in DNA capable of complexing with homologous ribosomal RNA and not occupiable by nonribosomal RNA from the same organism.

Materials and Methods.—Strain and preparation of materials: *E. coli* strains BB and A-155, a uracilless derivative of *B* were used in the present study. The methods of growth, labeling, counting, CsCl density gradient centrifugation, and the preparation of the following materials are detailed in the earlier papers;[1–3] heat-denatured DNA, free of RNAase; P^{32} and H^3-labeled 23S RNA free of "informational" RNA. The methods of assaying for RNAase activity and sensitivity of RNA-DNA complexes to nuclease degradation are the same as used in the earlier[1] study.

"Step-down" labeling of cells for informational RNA preparations: The procedure used is essentially that of Hayashi and Spiegelman.[3] Logarithmically growing *E. coli* A-155 cells in nutrient broth were collected by centrifugation, washed twice

in SC minimal broth, and resuspended in the minimal medium to an $O.D._{660}$ of 0.360. The cells were aerated for 10 min at 37°C; then 1.48 μg/ml of H^3-uridine was added (New England Nuclear Corporation, 3.28 c/mM) and aeration at 37°C continued for 10 min. Isotope incorporation was halted by pouring the cultures into 1/3 their volume of frozen, crushed minimal medium. The cells were collected by centrifugation, then concentrated 20-fold in TM buffer. The RNA was purified and fractionated in linear sucrose gradients by the methods described previously.[1, 3] The fractions corresponding to 8–12S were pooled, concentrated, and used in the experiments described.

Experimental Results.—A. *Saturation curve of the ribosomal RNA-DNA complex:* The detection of a saturation plateau requires the performance of experiments involving the following steps: (1) Mixtures containing fixed amounts of heat-denatured DNA and varying amounts of labeled ribosomal RNA are exposed to a slow cool from 55°C to 30°C; (2) The resulting products are then subjected to equilibrium density centrifugations in CsCl solutions to separate free RNA from that which is complexed to DNA. The sorts of hybrids observed are exemplified by the two cases in Figure 1. It will be seen that at the lower level of input, most of the RNA in the reaction mixture is complexed to the DNA. The proportion of the input fixed decreases as the concentration of RNA is increased.

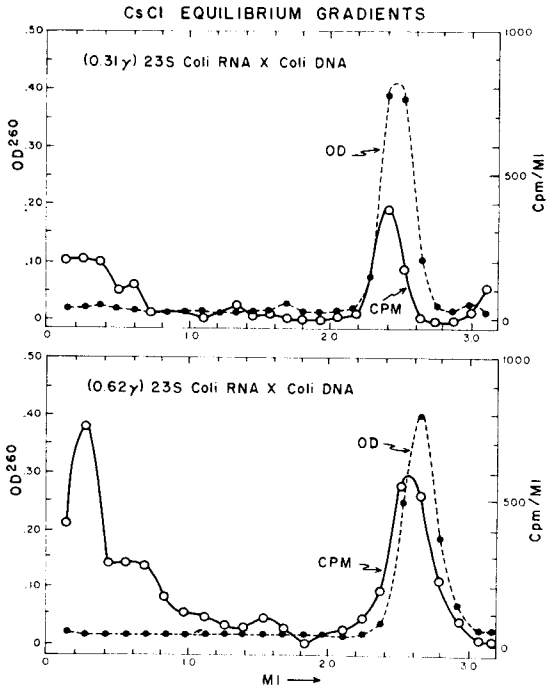

FIG. 1.—CsCl equilibrium density gradient profiles. Buffer used was TMSV (0.03 *M* tris, pH 7.3; 0.001 *M* $MgCl_2$; 0.3 *M* NaCl 0.005 *M* EDTA. Both contained 71 μg per ml of heat-denatured *E. coli* DNA. Upper contained 0.31 μg and the lower 0.62 μg of P^{32}-23S RNA (6.7×10^4 cpm/μg) of *E. coli*. Reaction mixtures slow (*ca.* 13 hr) cooled from 55°C to 33°C. Mixtures were then brought to a density of 1.73 with CsCl and a total volume of 3 ml. They were centrifuged for 52 hr at 33,000 rpm and 25°C.

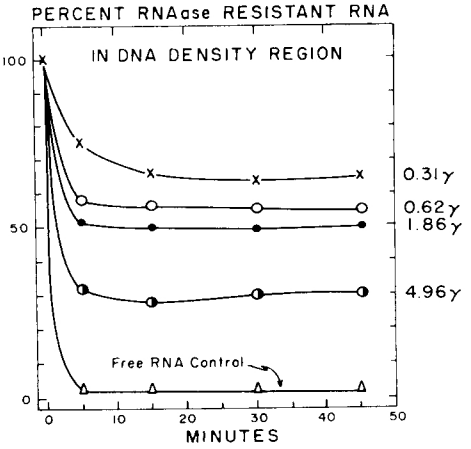

FIG. 2.—RNAase resistance of RNA-DNA complexes. 109 μg/ml of heat-denatured *E. coli* DNA were slow-cooled in TMSV with the indicated concentrations of *E. coli* 23S-P^{32}-RNA (6.7×10^4 cpm/μg). After separation of hybrids by centrifugation as in Figure 1, sensitivity to RNAase was examined as described previously.[1]

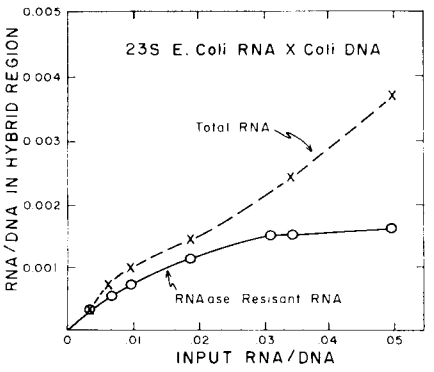

FIG. 3.—Saturation curves of total and RNAase-resistant counts in DNA region of CsCl equilibrium gradients. Details of incubations and centrifugation are as noted in Figures 1 and 2.

From our previous experience[1] with ribosomal RNA, it might be expected that nonspecific binding involving incidental coincidences over small regions will inevitably complicate such experiments. Adventitious complexing of this sort could become quantitatively serious at high levels of RNA input. Fortunately, as has been shown,[1] sensitivity to RNAase permits a ready distinction between nonspecific and specific complexes. This same device was used in the present study. Fractions in the hybrid regions of the density gradients were pooled, dialyzed, and exposed to RNAase.[1] The results obtained for four levels of RNA are detailed in Figure 2. In each case, free P^{32}-labeled RNA was included as an internal control in the reaction mixture. The behavior of only one control is depicted since they were all identical. It will be noted that the free RNA is almost completely solubilized in 5 min, whereas the H^3-labeled RNA from the hybrid regions exhibit RNAase-resistant residues of varying percentages. The data of Figure 2 are in agreement with what would have been expected from increasing amounts of nonspecific complexing at higher concentration levels of RNA. The greater the input of RNA, the larger is the proportion of the counts in the hybrid region which are found to be sensitive to RNAase.

A composite summary of these and similar experiments are plotted in Figure 3. We have here a comparison of the total RNA found in the DNA density region with that which resists degradation by RNAase. The behavior of these two are strikingly different. The total RNA shows no signs of saturation, whereas the resistant residue clearly approaches a plateau, corresponding approximately to an RNA: DNA ratio of 0.0015. The existence of this maximal level is consistent with the view that the DNA contains a restricted region capable of specifically complexing with ribosomal RNA.

B. *Competitive and noncompetitive interaction in the course of hybrid formation:* Competition experiments can also be used to reveal the existence of a localized specific interaction between ribosomal RNA and its homologous DNA. In the present system this can be realized by using two identifying isotopic labels on different RNA preparations. If the two RNA molecules are competing for the same site and the total concentration is at or near the saturation level, one label will displace the other as its proportion is increased in the reaction mixture. If they do not compete for the same site, fixation to the DNA of one will be essentially indifferent to the presence of the other.

To examine questions of this nature, the following types of experiments were performed with *E. coli* RNA and DNA. Mixtures containing fixed levels of P^{32}-23S RNA, heat-denatured DNA, and varying amounts of H^3-23S RNA were incubated and then centrifuged in CsCl. The amounts of P^{32} and H^3-labeled RNA fixed in the DNA and resistant to enzyme were then determined. For purposes of comparison, similar experiments were carried out with H^3-informational RNA (8–12S) replacing the H^3-23S RNA in the mixtures. The informational variety was chosen since it was known to complex well with DNA and thus provides a test for the specificity of the ribosomal combination with DNA.

Figure 4 gives two examples of the hybrid regions observed in CsCl gradients.

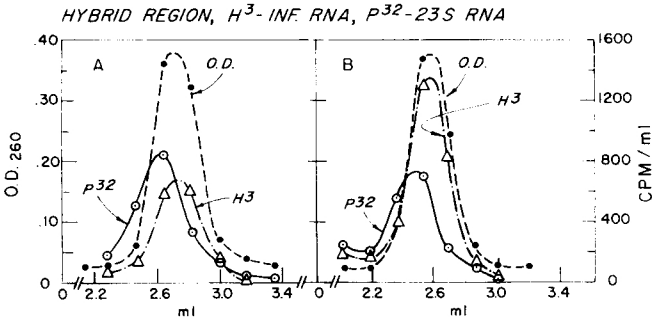

FIG. 4.—Hybrid regions in CsCl gradients of mixtures containing ribosomal and informational RNA. Both incubation mixtures contain 63 μg/ml of heat-denatured *E. coli* DNA and 2.66 μg/ml of *E. coli* P^{32}-23S RNA. (A) contained in addition 2.9 μg of H^3-informational RNA and (B) 5.7 μg of H^3-informational RNA per ml. Details of incubations and centrifugations are as in Figures 1 and 2.

Both types of nucleic acids have hybridized with, however, an interesting and consistent difference. The H^3-labeled informational RNA appears to be symmetrically distributed among the denatured strands of the DNA. This is clearly not the case with the ribosomal complex which is distinctly displaced toward the heavy side of the mean density of denatured DNA. The likely significance of this will be briefly noted in the *Discussion*.

Again, to avoid confusion with irrelevant complexes, the fractions in the hybrid regions were pooled and the RNAase-resistant residue of complexed radioactivity determined. Figure 5 summarizes the resulting data. There is clear evidence (Fig. 5*A*) of displacement of the P^{32}-labeled ribosomal RNA as more H^3-labeled RNA of the same kind is incorporated into the complex. Further, a saturation

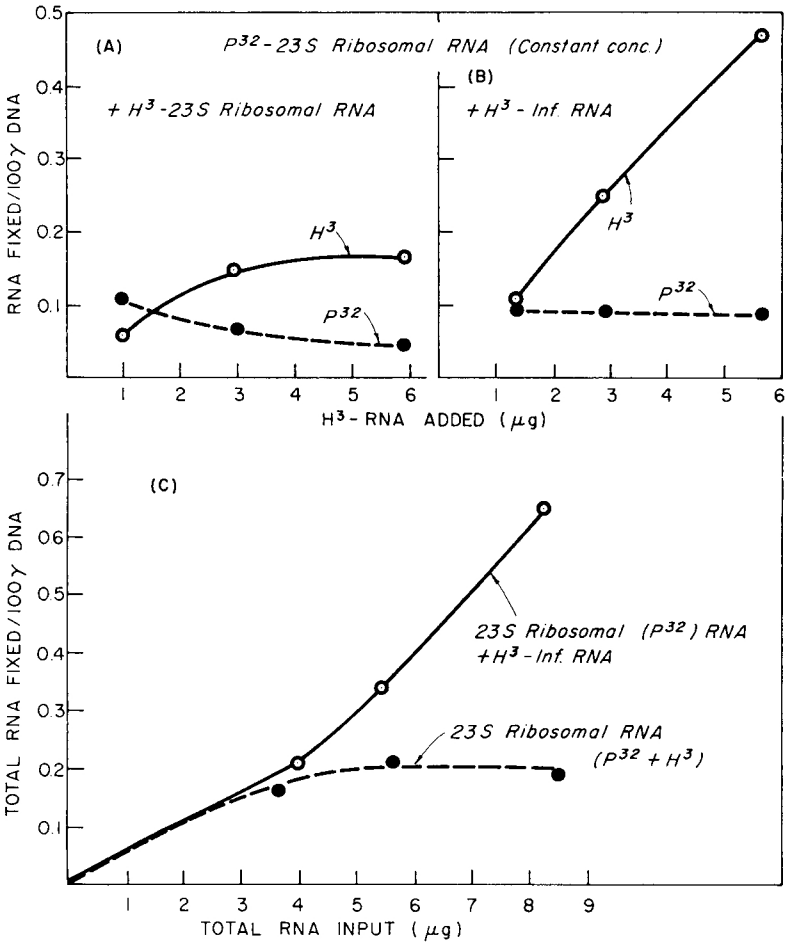

FIG. 5.—Tests for competitive interactions in mixtures of ribosomal and informational RNA molecules. (A) All mixtures contained 63 μg/ml of heat-denatured *E. coli* DNA and 2.66 μg/ml of *E. coli* P^{32}-23S ribosomal RNA. H^3-23S ribosomal RNA of *E. coli* varied as shown. (B) DNA and P^{32}-RNA same as (A) except that H^3-informational RNA was added in the amounts indicated. (C) The same preparations as (A) and (B). The plot here is total (P^{32} + H^3) RNAase stable counts per 100 μg of DNA as a function of total input of RNA. All incubations during slow cool and equilibrium centrifugations were carried out as described in Figure 1.

plateau in the H^3-RNA complexed is observed within the concentration range tested. On the other hand, when the H^3-label is on informational RNA, its hybridization with the DNA has no effect on the ability of the DNA to combine with the P^{32}-labeled ribosomal material (Fig. 5*B*). In addition, no evidence of saturation is observed with the informational variety within the concentration range tested. This difference in saturation behavior is more clearly illustrated in Figure 5*C* in which the total (P^{32} + H^3) RNA fixed per 100 γ of DNA is plotted against the total RNA in the mixture. When the ribosomal varieties alone are present, the sum of

the two labels fixed approaches a plateau—a phenomenon completely absent in the mixture of informational and ribosomal RNA.

These experiments provide additional evidence supporting the contention that ribosomal RNA specifically complexes with a restricted region not occupiable by nonribosomal RNA from the same organism.

Discussion.—The use of double labeling combined with equilibrium centrifugations in density gradients and enzyme sensitivity permitted the accumulation of data which leads to the conclusion that DNA does contain a localized sequence complementary to ribosomal RNA. The nature of the data obtained in the present and previous investigation[1] may be summarized as follows: (1) The RNA complexed in a hybridization test is ribosomal as demonstrated by the base composition of the hybridized material. (2) Complex formation stable to RNAase occurs only with homologous DNA. (3) Saturation of homologous DNA with ribosomal RNA in a complex stable to RNAase occurs when approximately 0.2 per cent of the DNA is occupied. (4) Competition for a specific site was established by using two identical ribosomal RNA preparations distinguishable by isotopic labeling. Under the same conditions, nonribosomal RNA from the same organism does not compete.

It will not escape the attention of the reader that certain problems of obvious interest have not received explicit mention. We may briefly cite a few. First, all the experiments described have employed only the 23S ribosomal component. This raises the obvious question of sequence similarity with the 16S variety. Further, no details are reported on heterologous tests among the bacteria, a point of obvious interest in view of the similarity of the ribosomal RNA base compositions. These, and related problems have been the subject of continuing investigations by the methods developed in the present investigation. The results will be reported in separate communications.

We may perhaps briefly allude to one point centering around the significance of the fact that ribosomal RNA saturates at a level corresponding to approximately 0.2 per cent of the DNA in *E. coli*. In view of the complicated operations required to obtain this number, its exact value must not be taken too seriously, and certainly not until similar determinations are reported with other organisms. However, values similar to this have been repeatedly obtained in independent experiments, and it would seem that it is certainly correct as to order of magnitude. This, however, is about 10 times greater than one would calculate from the DNA content per nucleus[4] and the assumption that there is only one complementary sequence per genome. We conclude, therefore, that there must be repeating units of this kind. They could be similar in sequence, or identical, depending on whether the population of ribosomal RNA molecules are identical or different. There is one highly suggestive observation which leads us to conclude that, whichever is the case, these repeating units are not scattered throughout the genome, but contiguous. A persistent pecularitiy observed with homologous ribosomal RNA-DNA hybrids (Fig. 1 and P^{32} curves of Figs. 4*A* and 4*B*) is the pronounced displacement of the complex towards the heavy side of the mean density of the heat-denatured DNA. This displacement is not observed with the heterologous complexes.[1] Furthermore, it is not seen with informational RNA-DNA hybrids at this level of RNA input (H^3-curves of Figs. 4*A* and 4*B*). The simplest interpretation for this shift is to assume that the repeating units complementary to ribosomal RNA are contiguous

and that any DNA strand which has one such sequence is likely to have another. Consequently, this selected set of strands complex with several ribosomal RNA molecules, resulting in the observed increase in density.

Summary.—The experiments reported offer further evidence for the presence in DNA of a sequence complementary to ribosomal RNA. Saturation experiments suggest that the particular region involved corresponds to between 0.1 and 0.2 per cent of the total genome. Competition for a restricted DNA region can be exhibited by the use of variously labeled homologous ribosomal RNA. Nonribosomal RNA from the same organism does not compete for the same site. The amount of ribosomal RNA complexed per unit of DNA at saturation suggests a number of repeating, similar, or identical sequences. Further, the density shift of the hybrids suggests that these units are not scattered but contiguous in the DNA structure.

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† Predoctoral fellow trainee in Molecular Genetics (USPH 2G-319).

[1] Yankofsky, S., and S. Spiegelman, these PROCEEDINGS, **48,** 1069 (1962).

[2] Hall, B. D., and S. Spiegelman, these PROCEEDINGS, **47,** 137 (1961).

[3] Hayashi, M., and S. Spiegelman, these PROCEEDINGS, **47,** 1564 (1961).

[4] Barner, H., and S. S. Cohen, *J. Bacteriol.*, **72,** 115 (1956).